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Extended Abstract

Biodegradation of 5-(Hydroxymethyl)-furfural and Furan Derivatives †

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Abstract: Furfural and 5-hydroxymethylfurfural (HMF) are degradation products of lignocellulose during pretreatment operations. Furfural compounds are a group of chemical compounds whose common thread is an aldehyde group attached to a furan ring, and they constitute a problem for the development of second-generation biofuels because they act as fermentation inhibitors of the lignocellulose hydrolysates. Up to date, very few bacteria have been described to be able to eliminate them. The objective of this work was to isolate and characterize bacterial strains able to use, as the sole carbon source, 5-(hydroxymethyl)-furfural (HMF) and furan derivatives.

Keywords: *Pseudomonas*; furan derivatives; biodegradation

1. Introduction

The production of biofuels from lignocellulosic residues constitutes a viable option for reducing the greenhouse effect and for providing an alternative to fossil fuels [1]. A thermochemical pretreatment is necessary to release fermentable sugars during the production of biofuels from lignocellulosic residues to alter the structure of cellulosic biomass to make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars [2]. Although necessary, numerous by-products, including furan derivatives, weak acids, and phenolic compounds, are generated in the pretreatment step [1]. Some degradation products from lignocellulose pretreatment strongly inhibit the activities of cellulolytic enzymes and ethanol fermentation strains, for this reason the removal of the inhibitor compounds is very important for the biotransformation processes [3]. Furfural and 5-hydroxymethylfurfural (HMF) are among these degradation products of lignocellulose during [4]. Furfural compounds are a group of chemical compounds whose common thread is an aldehyde group attached to a furan ring, and they constitute a problem for the development of second-generation biofuels because they act as fermentation inhibitors of the lignocellulose hydrolysates [2]. Investigations on optimization of pretreatment process to minimize the formation of furfural and HMF are being addressed, however, this approach is non-optimal [5]. Alternatively, microbial conversion of these compounds to carbon and energy sources would be much more desirable [3]. Although several microorganisms are known to degrade furanic compounds, the variety of species is limited mostly to Gram-negative aerobic bacteria, with a few notable exceptions [3]. Koopman, Wierckx, and co-workers reported the discovery of a metabolic pathway in the soil isolate *Cupriavidus basilensis* HMF14 [6]. Since then several

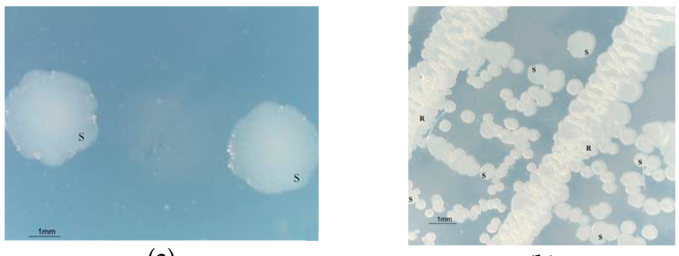
biodetoxification strains had been applied to degrade the lignocelluloses-derived inhibitor compounds [4,7–11].

2. Materials and Methods

Bacterial isolation. Bacterial strains were isolated after an enrichment cultivation procedure. The medium used was the M9 minimal medium prepared at pH 7.5, with with ammonium chloride (5 mM) and HMF (5 mM) as the sole added nitrogen and carbon sources, respectively. The medium was inoculated with field ashes samples of pruning wastes and incubated in an Erlenmeyer flask at 37°C in a rotatory shaker. After 2 weeks, the process was repeated 6 times by reinoculation in fresh medium with 1% of the previously grown culture. Samples of the enriched culture were plated on Luria-Bertani (LB) medium solidified with 1.8% Bacto agar (BD Difco, Hampton, NH, USA), and individual colonies were purified and tested for axenic growth in liquid cultures with HMF as the sole carbon source. Two types of colonies were able to assimilate HMF, thus producing an axenic culture from which a single colony was selected, obtained in pure culture, and kept for further analysis. Culture media. The bacterial strains were grown either in M9 minimal medium adjusted to pH 7.5 on a rotatory shaker at 230 rpm and 30 °C. Unless otherwise stated ammonium chloride (5 mM) was used as the nitrogen source. The appropriate carbon sources were added from sterilized stocks at the indicated concentrations. Bacterial growth was monitored by determining the absorbance at 600 nm. Taxonomic position and analysis of 16S rRNA gene sequence of the bacterial isolates. Genomic DNA from the bacterial strains was obtained by using a Puregene kit B (Qiagen, Hilden, Germany) following the instructions of the manufacturer. Gene encoding the small ribosome subunit (16S rRNA) was sequenced using services provided by Sistemas Genómicos (Valencia, Spain). After a BLAST analysis of the sequences, the isolates were tentatively identified.

3. Results

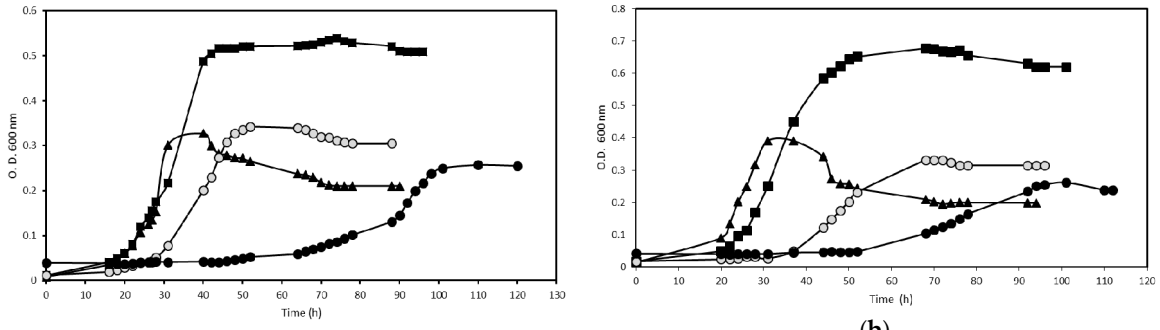
In the present study, we have isolated from field ashes samples of pruning wastes, after selective enrichment cultures two bacterial strains able to use 5-(hydroxymethyl)-furfural (HMF) and furan derivatives as the sole carbon source in minimal medium with ammonium chloride as nitrogen source under aerobic conditions. By comparison of the 16S RNA gene sequence with existing sequences, the isolated strains were identified as *Pseudomonas* spp. 3A strain and *Pseudomonas* spp. 4A strain. Two distinct colonial types, R-type and S-type, appeared among the bacterial isolates when they were cultivated on solid Agar medium (Figure 1). S (smooth) colonies are circular, smooth or continuous edge, shiny surface; and they easily dispersed in water, giving homogeneous suspensions. R (rough) colonies often have a wavy or scalloped edge, they are flat, rough texture and matt appearance, and they develop a characteristic wrinkled appearance and do not disperse well in water. This fact has been described as colonial dissociation [1]. Colonial dissociation depended on the culture medium. *Pseudomonas* spp. 3A and Pseudomonas spp. 4A cultured in rich medium (LB-Agar) formed two types of colonies (S) and (R) (Figure 1b) although the type R colony was predominant. However, cultures in M9-HMF-Agar medium only gave rise to S-type colonies (Figure 1a).



(**a**) (**b**)

Figure 1. *Pseudomonas* spp. 4A strain colonial dissociation in LB-agar plates. (**a**) Smooth colonies (S), and (**b**) a mixture of Rough (R) and Smooth colonies (S).

In order to optimize the process and to determine the range of HMF concentrations that both strains were able to assimilated, cultures with increasing concentrations of HMF were used. Both strains were able to assimilate up to 10 mM of HMF, although concentrations higher than 5 mM produced an increase in the latency phase of the cultures probably due to the toxicity of the substrate. The maximum growth values were greater than 10 mM > 7.5 mM > 5 mM > 2.5 mM, while the latency phase values were inverse. These results could be explained because when the molarity of the compound (HMF) increases as well as the concentration of the carbon source, but also the toxicity of the medium, so more time to consume the carbon source is needed. The growth rate of *Pseudomonas* spp. 3A and *Pseudomonas* spp. 4A was maximum in media with furoic acid and HMF. However, the cellular yield, determined as the final absorbance of the culture was higher with HMF (Figure 2), which is not surprising since this compound contains an additional carbon. The lag phase was quite reduced with furoic acid, and showed an important increase in media with furfural (Figure 2). *Pseudomonas* spp. 4A (Figure 2a) achieved a greater maximum growth than strain 3A (Figure 2b) in all furanic compounds. In *Pseudomonas* spp. 4A the lag phase was also lower, except with furfuryl alcohol.



(**a**) (**b**)

Figure 2. Growth of *Pseudomonas* spp. 4A (**a**) and *Pseudomonas* spp. (**b**) with 5 mM of HMF (■), furoic acid (▲), furfuryl alcohol ( ) or furfural (●) as the sole carbon source. Cells were inoculated in M9 media at pH 7.5 containing NH_4Cl (5 mM) as the nitrogen source. At the indicated times, the increments of cell growth of each culture were measured. A no inoculated flask containing culture medium was used as a control (result not shown). The experiment was carried out in triplicate obtaining similar results.

4. Discussion

The bacterial strains *Pseudomonas spp*. 3A and 4A able to use (HMF) and furan derivatives were isolated after an enrichment cultivation procedure. *Telluria* spp., *Arthrobacter* spp. and *C. basilensis* HMF14, able to assimilate HMF, were also isolated by selective enrichment of soil samples from a botanical garden and water samples from a lake [12]. Isolated showed colonial dissociation depending of culture media. In *Streptomyces aureofaciens* galactose induces the dissociation of colonies [13].This morphological heterogeneity in bacterial colonies dependent on culture conditions has also been described in the case of *Pseudomonas stutzeri* [14]; in fact the R type colonies of *P. stutzeri* and those of the bacteria isolated in this work have a very similar appearance (Figure 1). Colonies generally resemble craters with elevated ridges that often branch and merge, and they have been described as tenacious, with a coral structure [14]. Both strains (3A and 4A) were able to assimilate up to 10 mM of HMF. Something similar was described in *C. basilensis* HMF 14, although at a different concentration range (3–12 mM) [2]. The efficiency in the assimilation of furanic compounds of *Pseudomonas* spp. 3A (Figure 2b) and *Pseudomonas* spp. 4A (Figure 2a) could be explained from a bioenergetic point of view, since in a respiratory metabolism, the more oxidized the carbon source is (acid > aldehyde > alcohol) the less energy can be extracted from it; therefore both strains grew faster in furoic acid. Bacterial isolated grew slowly n cultured media with furfural, probably this growth is directly affected not only by the contribution of carbons but also by the toxicity of the compounds, being furfural the most toxic compound, followed by furfuryl alcohol, furoic acid and HMF.

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